Pergamon

PII: S0042-6989(97)00115-6

Vision Res., Vol. 37, No. 22, pp. 3089–3096, 1997

Printed in Great Britain
0042-6989/97 $17.00 + 0.00

The Primary Structure and Distribution of Killifish Visual Pigments

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Received 21 August 1996; in revised form 1 April 1997

Five cDNA fragments (KFH-R, -V, -G, -B and -Rh) encoding the putative visual pigments of killifish were isolated and sequenced. Judging from the deduced amino acid sequences, each cDNA falls into a different group of the five major families of vertebrate visual pigment genes. *In situ* hybridization localized the mRNA of KFH-R and -G to the principle and accessory members, respectively, of double cones. Visual pigment genes KFH-Rh, -B and -V were expressed in the rods, and the long and short single cones, respectively. It is suggested that the relationships between the cell types and their respective visual pigment gene groups may be a common pattern among teleost fishes. © 1997 Elsevier Science Ltd

Visual pigment cDNA cloning Cone cell arrangements *In situ* hybridization
Killifish *(Oryzias latipes)*

INTRODUCTION

Fishes have developed color vision systems that appear to be closely adapted to the species' photic environment (Loew & Lythgoe, 1978). The spectral sensitivity of a photoreceptor cell is due mainly to the absorption maximum of its visual pigment, which is closely related to its primary structure. Therefore, the primary structures of visual pigments may somehow be influenced by the habit of an animal and environment in which it lives.

Cone cells in many teleost fishes are morphologically distinguishable (see for example, Munz & McFarland, 1977) but their distinctions are poorly understood at the molecular level. Microspectrophotometric and immunohistchemical studies have led to the proposal that each cone cell type has its own visual pigment. However, the relationships between cell type and the visual pigment primary structure expressed in these cells have been clarified only for the goldfish (Johnson *et al.*, 1993; Raymond *et al.*, 1993; Hisatomi *et al.*, 1996).

Cone cells are often arranged in the teleost retina in species-specific mosaic patterns. Fish eyes are large relative to body size and these mosaic arrays are themselves enlarged during the early developmental stages through to the adult. Photoreceptor cells probably differentiate at the ciliary marginal zone to produce the mosaic (Johns & Easter, 1977; Johns, 1977; Negishi *et al.*, 1990a Negishi *et al.*, 1990b; Fernald, 1991). The mechanism responsible for constructing the mosaic is still unclear, but visual pigments are candidate molecular markers for following the differentiation of teleost photoreceptor cells (Stenkamp *et al.*, 1996).

The photoreceptor cells of the killifish or medaka *(Oryzias latipes)* can be categorized morphologically into five types: rods, double cone principle and accessory members, long single cones, and short single cones. The cone cells are reported to be arranged in a square mosaic pattern (Ohki & Aoki, 1985). The spectral sensitivities of these cells have not been clarified so far but the cDNA isolation and sequence determination of their visual pigments may serve as aids to understanding, at the molecular level, the evolution of visual pigments and the genetic programs for photoreceptor differentiation. In this paper we report on the isolation and characterization of cDNAs encoding the killifish putative visual pigments, and the localization of their transcripts by *in situ* hybridization. Our results suggest that the relationships between photoreceptor cell types and the gene groups of visual pigments expressed in photoreceptor cells may be common to many teleost fishes.

MATERIALS AND METHODS

Isolation of killifish genomic DNA and cDNA fragments encoding the putative visual pigments

Twenty adult killifishes (*Oryzias latipes*, about 3 cm in length) were obtained from a local supplier and genomic DNA was isolated from the whole animals, as described by Maniatis *et al.* (1989). Poly(A) RNA was isolated from about 100 killifish retinas, using a Quick Prep *Micro*

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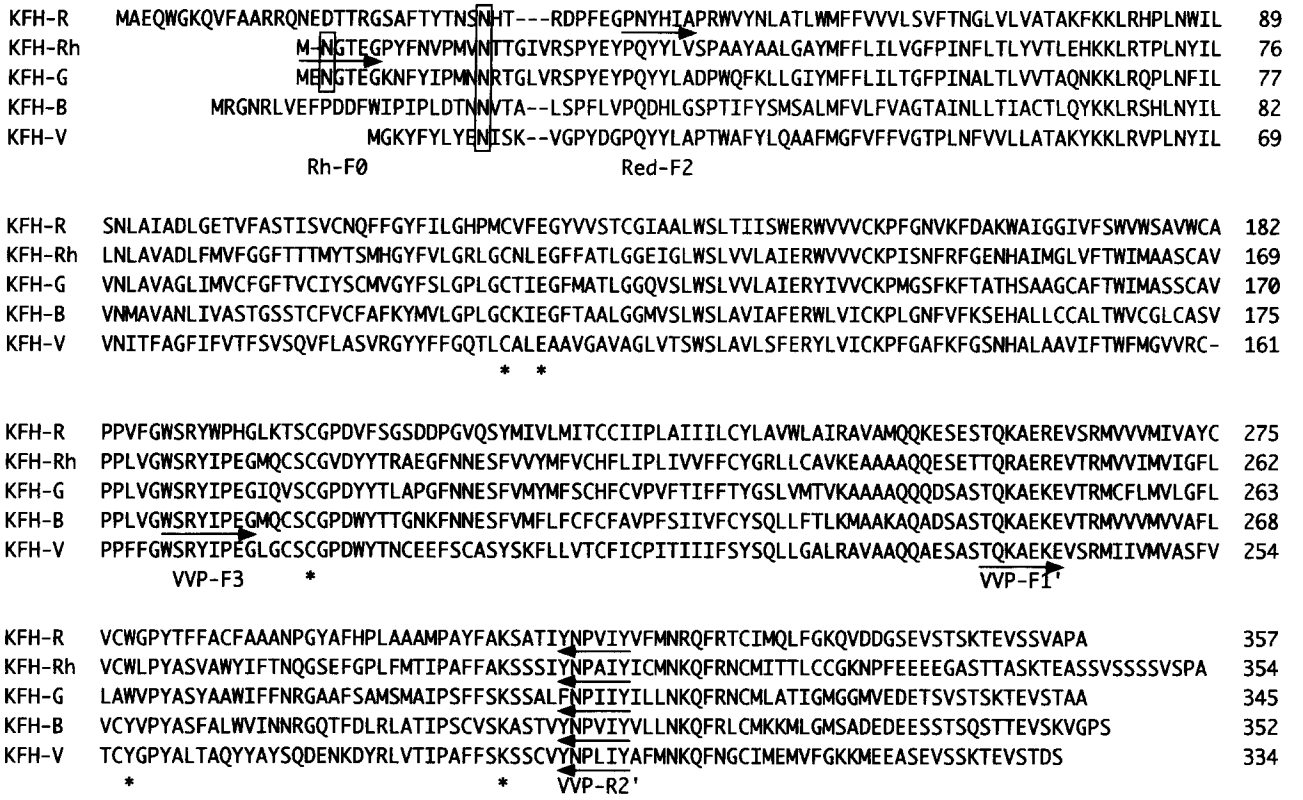

FIGURE 1. Deduced amino acid sequences of the putative visual pigments of killifish. Arrows represent the direction and amino acid sequences corresponding to degenerate oligonucleotides RhF0, RedF2, F3, F1′ and R2′. Boxes represent the amino acids probably glycosylated, and asterisks those apparently conserved in all vertebrate visual pigments (see text). The sequences presented have been deposited in the DDBJ data base (Accessions Numbers: AB001602–AB001606).

mRNA Purification Kit (Pharmacia), and double stranded cDNA was synthesized using Superscript II reverse transcriptase (Gibco BRL) with random hexa-oligonucleotides and oligo $dT_{12\text{-}18}$ as primers (Pharmacia).

Degenerate oligonucleotides (RedF2, F3, F1′, RhF0 and R2′, corresponding to amino acid sequences PNYHIAP, WS(R/A)(Y/F)(I/V/W)P(E/H)G, TQKAEKE, MNGTEG and YNP(I/V)VY, respectively) were synthesized and used as primers for polymerase chain reactions (PCRs). Genomic DNA and cDNA fragments encoding killifish visual pigments were amplified as described in Hisatomi *et al.* (1994), and used as probes for screening a killifish retinal cDNA library.

Construction and screening of killifish retinal cDNA library

A cDNA library was constructed using a Lambda ZAPII *Eco*RI/CIAP Vector Kit (Stratagene). Briefly, double-stranded cDNA was ligated with *Eco*RI–*Not*I adaptor and inserted into an *Eco*RI site of Lambda-ZAPII. The cDNA was size-selected with Size-Sep 400 spun columns (Pharmacia) before and after the addition of the adaptor, and packaged into Giga-Pack II gold (Stratagene). The amplified fragments encoding killifish visual pigments were used as probes for high stringency screening (hybridization: 50% formamide at 48°C; washing: 0.2 × SSC 0.1% SDS at 55°C; Kayada *et al.*, 1995). Low stringency hybridizations were carried out in hybridization buffer containing 10% formamide at 45°C, and washed with 2 × SSC 0.1% SDS at 55°C.

Sequencing and data analysis

Positive clones were transformed into plasmids by an EXASSIST-SOLR system (Stratagene) and sequenced by an ordinal deletion method (Maniatis *et al.*, 1989). Sequencing reactions were carried out according to the cycle sequencing method, as recommended in the manufacturer's protocol (Applied Biosystems). All sequences were determined in both directions using an ABI-373A automatic DNA sequencer (Applied Biosystems). Evolutionary distances of the sequences (k) were calculated for 290 amino acids between the region corresponding to P16 and Q328 of killifish rhodopsin, using the proportion of different amino acids between the two sequences (p), with a correction for multiple substitutions of $k = -\ln(1 - p - p^2/5)$ (Kimura, 1983). A phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei, 1987), using Drosophila and octopus rhodopsin as outgroups (Zuker *et al.*, 1985; Ovchinnikov *et al.*, 1988a).

In situ *hybridization*

DNA fragments were cloned into a pGEM-3Zf(+)

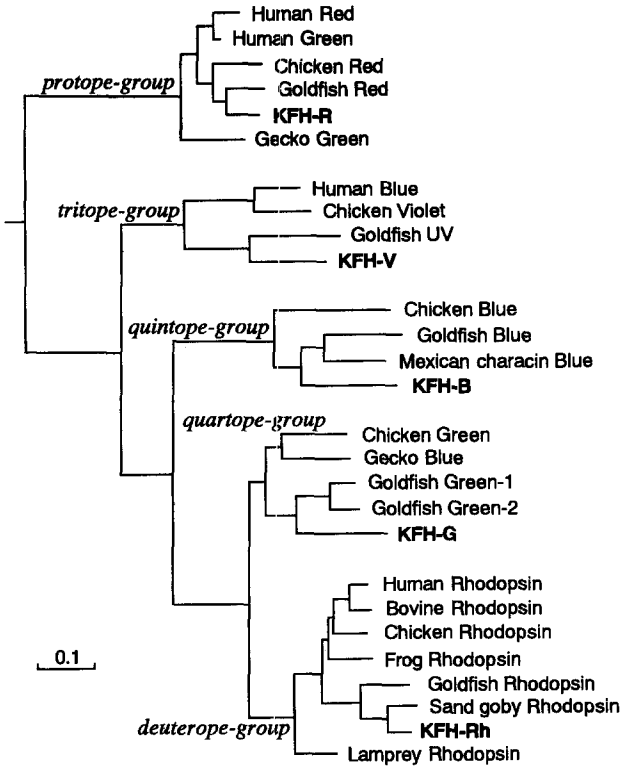

FIGURE 2. A phylogenetic tree of vertebrate visual pigments. Amino acid sequences are taken from the literatures: human cone pigments (red, green and blue, Nathans *et al.*, 1986) and rhodopsin (Nathans & Hogness, 1984); chicken cone pigments (red, Tokunaga *et al.*, 1990; green, blue and violet, Okano *et al.*, 1992) and rhodopsin (Takao *et al.*, 1988); goldfish pigments (red, green-1, green-2, blue and rhodopsin, Johnson *et al.*, 1993; UV, Hisatomi *et al.*, 1996); gecko pigments (green and blue, Kojima *et al.*, 1992); characin blue (Yokoyama & Yokoyama, 1993); frog rhodopsin (Kayada *et al.*, 1995); sand goby rhodopsin (Archer *et al.*, 1992); and lamprey rhodopsin (Hisatomi *et al.*, 1991).

plasmid vector (Promega), and plasmids were linearized, with appropriate endonucleases, to generate templates for the preparation of antisense cRNA probes. Antisense cRNA riboprobes (500–1000 bases in length) were synthesized by run-off transcription from the SP6 promoter with digoxigenin-UTP, as recommended in the manufacturer's protocol (Boehringer Mannheim).

Cryosections of light-adapted killifish retinas were prepared as described in Barthel & Raymond (1990). Briefly, enucleated eyes were fixed in 4% paraformaldehyde, infiltrated with 20% sucrose in phosphate buffer (PB: 100 mM sodium phosphate, pH 7.4), and embedded in 33% OCT compound diluted with 20% sucrose in PB. 3–10 μm retinal cryosections were prepared and stored at −80°C until use. The methods for *in situ* hybridization of retinal cryosections are as described in Barthel & Raymond (1993) and Raymond *et al.* (1993), with slight modifications as follows. Sections stored at −80°C were thawed, rehydrated, incubated in 10 μg/ml proteinase K (Boehringer Mannheim) for 2–3 min at 37°C, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine. After dehydration, sections were hybridized at 65°C for 16 hr with 0.1 μg/ml (final concentration) cRNA probes in a hybridization solution (Rosen & Beddington, 1993), washed with 50% formamide in 2 × SSC (pH 4.5) at 65°C for 2 hr, treated with 10 μg/ml (final concentration) RNase A (Boehringer Mannheim) at 37°C for 1 hr, and blocked with 0.5% blocking solution (Boehringer Mannheim) at 0°C for 30 min. Slides were incubated at 4°C for 12–24 hr with anti-digoxigenin antibodies conjugated with alkaline phosphatase (AP) and the hybridization signal was detected by incubation for 0.5–16 hr with 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate). Sections were rinsed with TE (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA) and coverslipped under 80% glycerol, then photographed with Nomarski optics.

RESULTS AND DISCUSSION

Isolation of cDNAs encoding visual pigments

Degenerate oligonucleotides (see Materials and Methods) corresponding to the amino acid sequences conserved in vertebrate visual pigments were made for the amplification of genomic DNA and cDNA fragments encoding killifish visual pigments. Four kinds of DNA fragments (KFH-R, -B, -V and -Rh) were amplified using R2′ as an anti-sense primer, and RedF2, F3, F1′ and RhF0 as respective sense primers. Using these amplified DNA fragments as probes, a killifish retinal cDNA library containing 10^6 independent clones was screened at high stringency. 0.6–1.0, 0.2, 0.04 and 1.5–2.0% of the plaques hybridized to KFH-R, -B, -V and -Rh probes, respectively. The cDNA library was screened again at low stringency, using as a probe a goldfish genomic DNA fragment, GFH-4 (the putative green-sensitive pigment of goldfish; Hisatomi *et al.*, 1994). About 0.06% of the plaques proved positive with this screening.

Positive clones for each probe were isolated and sequenced. Translational initiation codons were identified as the first ATG, and by comparison with those of other vertebrate opsins. KFH-R, -G, -B, -V and -Rh cDNA fragments were found to have 5′ non-coding regions of about 60, 60, 110, 15 and 50 bases, and 3′ non-coding sequences of about 150, 350, 80, 210 and 450 bases, respectively. The putative poly-adenylation signals were found 20–50 bases upstream from the start of the poly(A) tails.

The deduced amino acid sequences of killifish visual pigments

Figure 1 shows the deduced amino acid sequences of killifish visual pigments. The hydropathy plots of these sequences predicted seven transmembrane segments for each visual pigment (cf. bovine rhodopsin; Hargrave *et al.*, 1983). Amino acids typical of vertebrate visual pigments were found in the deduced amino acid sequences, that is: a lysine that could form the Schiff base linkage to the chromophore (Wang *et al.*, 1980); a glutamic acid that could serve as the Schiff base counterion (Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989; Nathans, 1990); sites for the disulfide bond (Karnik *et al.*, 1988); *N*-glycosylation site(s); and possible

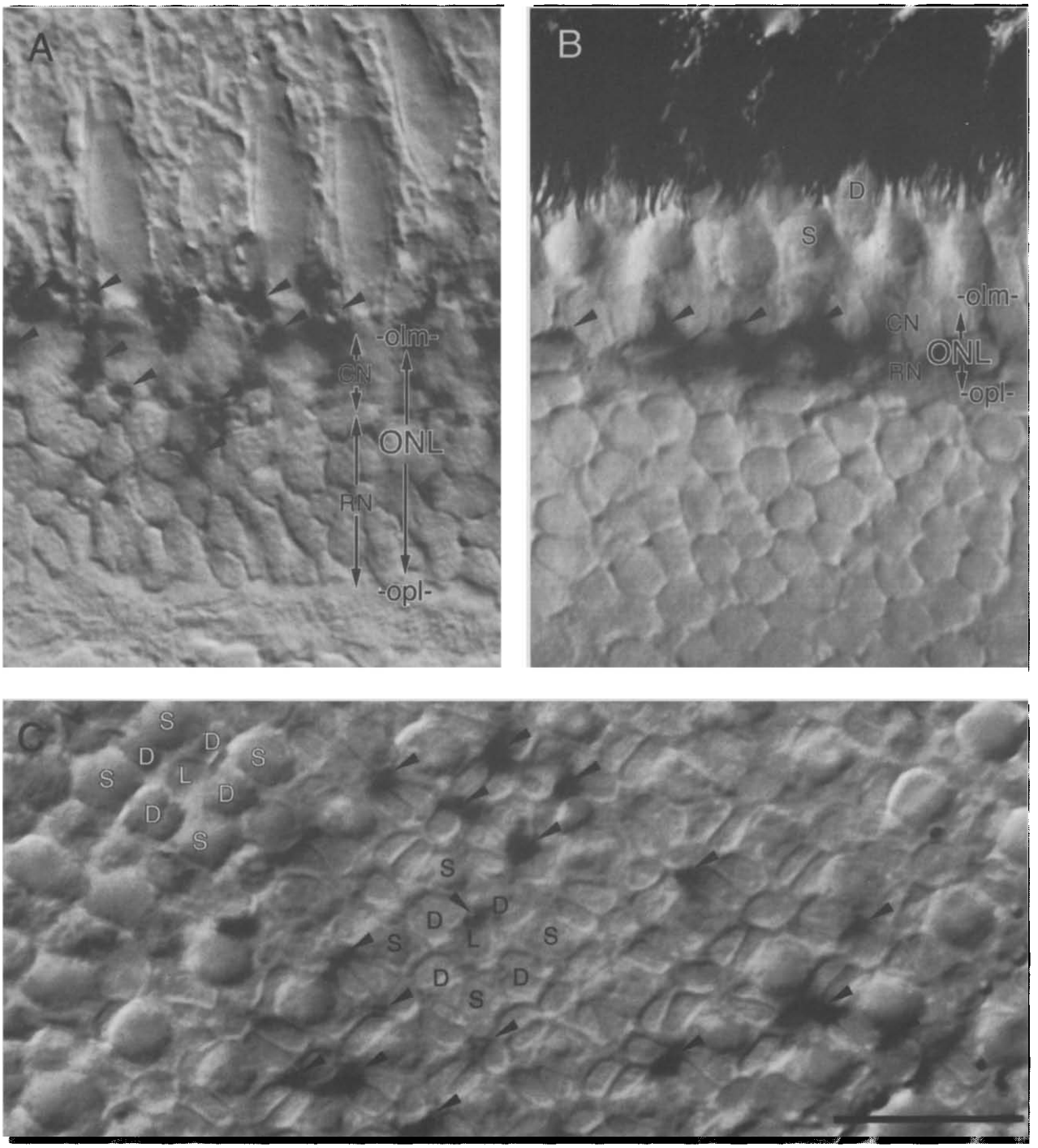

FIGURE 3. Localization of KFH-Rh mRNA in radial sections of adult (A) and 1-week-old fry (B) killifish retina. Arrowheads indicate the hybridization signals, and arrows indicate the layers in which rod nuclei (RN, inner side) and cone nuclei and rod myoids (CN, outer side) are present. (C) Localization of KFH-mRNA in a tangential section of 1-week fry retina. Mosaic patterns of different depths of cone cell layer are seen in the central (close to cone nuclei) and upper left (close to cone myoids) regions. Signals (arrowheads) are distributed randomly in the cone cell array. ONL, outer nuclear layer; olm, outer limiting membrane; opl, outer plexiform layer; D, double cone; L, long single cone; S, short single cone. Bar = 10 μm.

phosphorylation sites near the C-terminal (Ohguro *et al.*, 1994). These cDNAs therefore seem to encode functional visual pigments.

The deduced amino acid sequences of KFH-R, -G, -B, -V and -Rh show more than 60% identity with those of red-, green-, blue- and violet- (ultraviolet-) sensitive cone pigments and rhodopsin, respectively, of chicken and goldfish (Takao *et al.*, 1988; Tokunaga *et al.*, 1990; Okano *et al.*, 1992; Johnson *et al.*, 1993; Hisatomi *et al.*, 1996). A phylogenetic tree constructed by the neighbor-joining method (Fig. 2) leads us to deduce that these cDNAs can each be classified into one of the five gene groups (*protope*, *deuterope*, *tritope*, *quartope* and *quintope*) of vertebrate visual pigments (Hisatomi *et al.*, 1994). It is speculated that visual pigments in most vertebrates have evolved along five fundamental lines.

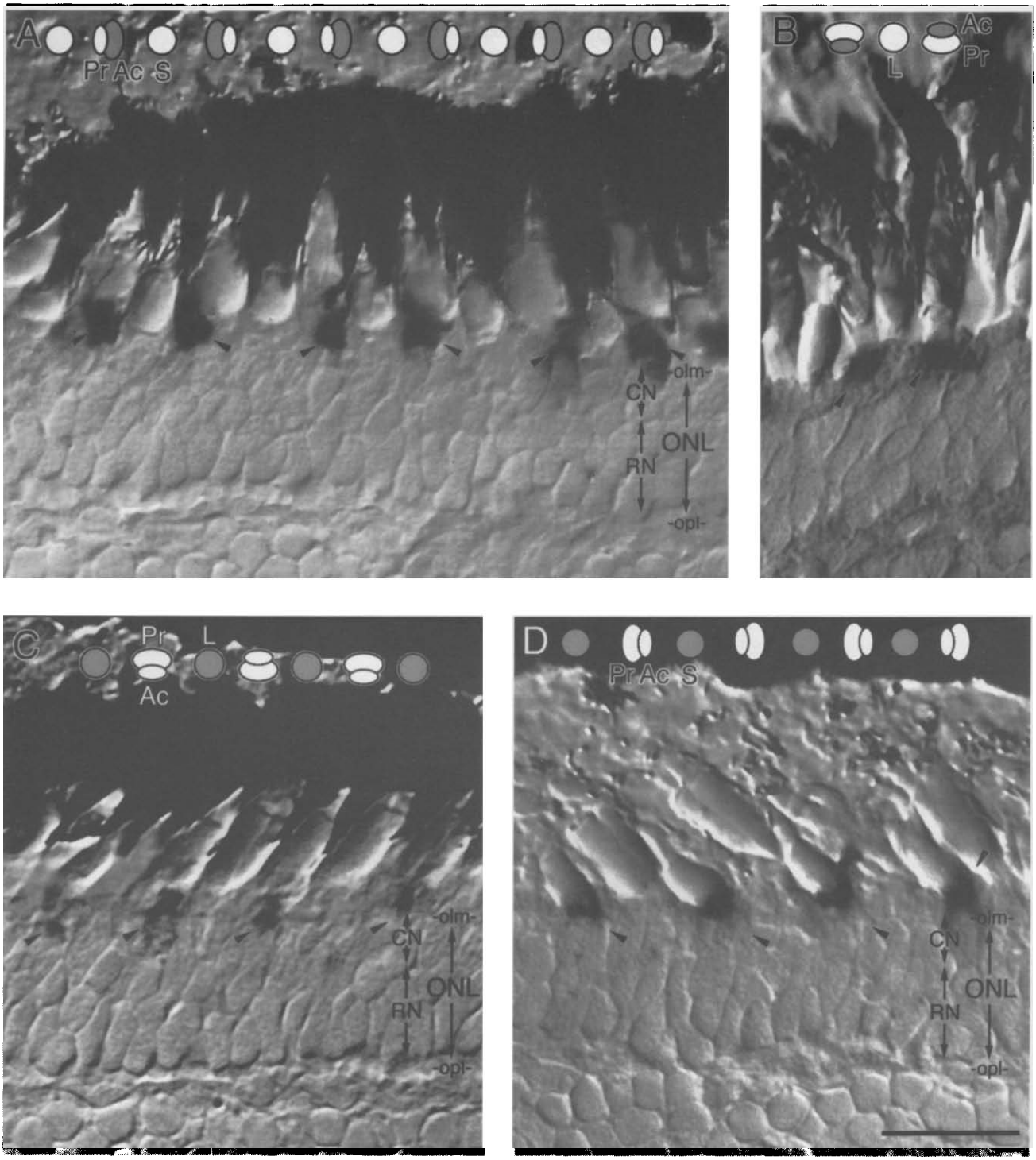

FIGURE 4. Localization of mRNA of KFH-R (A), KFH-G (B), KFH-B (C), and KFH-V (D) in radial sections of killifish retina. Hybridization signals (arrowheads) are localized to the myoid regions of these photoreceptor cells. Diagrammatic upper insets represent the arrangements of the cone photoreceptors in these sections, and cells hybridized with each mRNA are shaded. D, double cone (Pr, principle member; Ac, accessory member). Other abbreviations as in Fig. 3. Bar = 10 μm.

The spectral sensitivities of killifish photoreceptor cells have not been determined, but it seems likely that pigments encoded by KFH-R, -G, -B, and -V will probably be red-, green-, blue-, and violet- (or ultra-violet-) sensitive, on comparing the sequences to those of goldfish and other vertebrate visual pigments (Nathans *et al.*, 1986; Okano *et al.*, 1992; Johnson *et al.*, 1993; Hisatomi *et al.*, 1996). Interestingly, KFH-B has Y271, whereas other visual pigments belonging to the *quartope* group have a tryptophan residue at this position. A point mutant of bovine rhodopsin, W265Y, was reported to have an absorption maximum 15 nm shorter than that of the wild type (Nakayama & Khorana, 1991), suggesting that photo-sensitivity of the pigment encoded by KFH-B might be shifted to a shorter wavelength.

In bovine rhodopsin, it has been suggested that two cysteine residues, Cys322 and Cys323, are anchored in the cell membrane by palmitic acid esterification

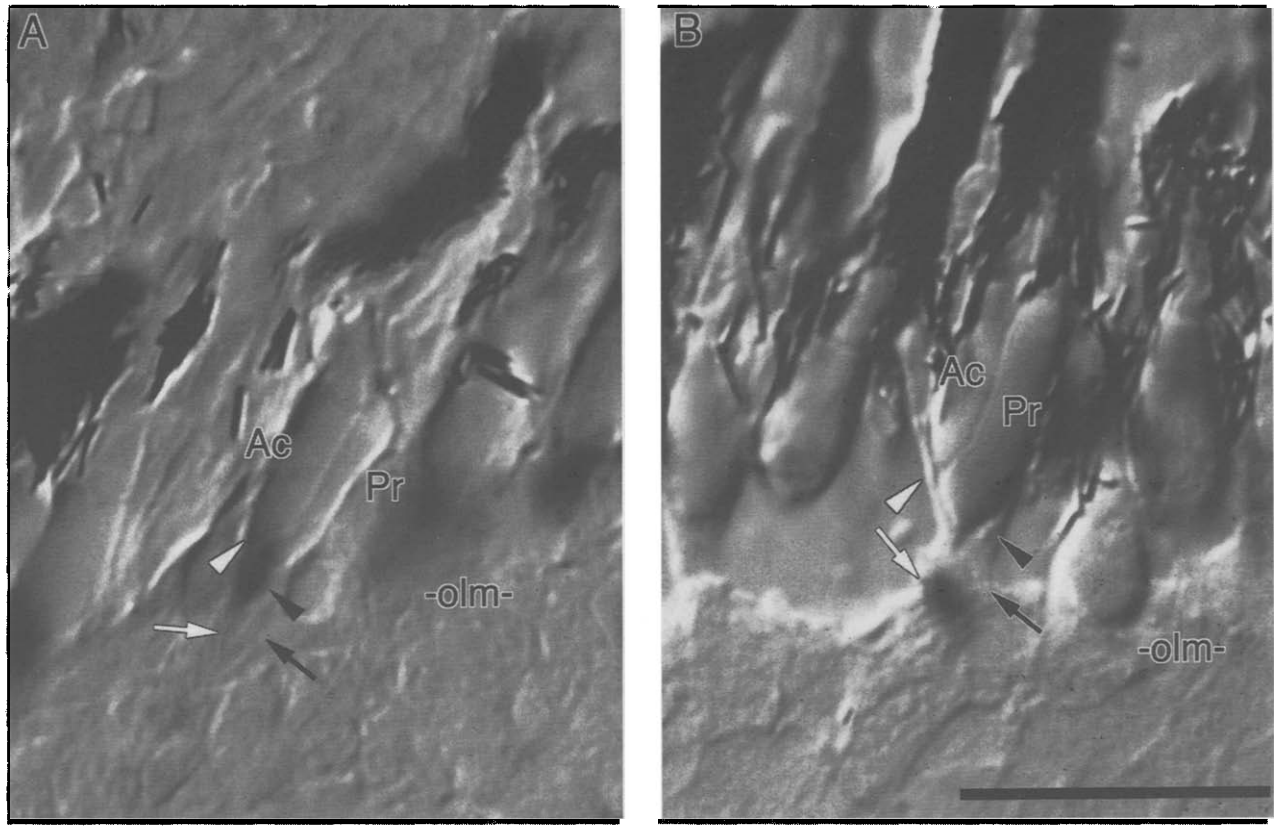

FIGURE 5. Subcellular localizations of KFH-R (A) and KFH-G (B) mRNAs in double cones. Arrows and arrowheads indicate the proximal and distal parts of the myoids of principle (filled) and accessory (open) members, respectively. Abbreviations as in Fig. 3. Bar = 10 μm.

(Ovchinnikov *et al.*, 1988b). Killifish cone pigments (KFH-R, -G, -B, and -V) do not have cysteine residues at corresponding positions. An inserted glutamine residue (Glu2) at the N-terminal of KFH-G, and the deletion of an amino acid residue at the end of the fourth transmembrane segment of KFH-V seem to have occured after the divergence between goldfish and killifish.

Distribution of visual pigment mRNAs in killifish photoreceptor cells

To assess the correspondence between the five prospective visual pigments and the five morphologically different types of photoreceptor cell present in killifish, we investigated the distribution of killifish visual pigment mRNAs by *in situ* hybridization. The digoxigenin-conjugated KFH-Rh cRNA probe recognized the outer half of the outer nuclear layer in light-adapted retina of adult killifish [Fig. 3(A)]. The patchy appearance of the signals was similar to that observed with *in situ* hybridization of the goldfish rhodopsin cRNA probe (Raymond *et al.*, 1993; Stenkamp *et al.*, 1996). Localization of KFH-Rh mRNA was also investigated in a radial section of 1-week-old fry retina [Fig. 3(B)]. The outer nucler layer of fry killifish retina can be distinguished into two layers in which rod nuclei (inner side) and cone nuclei and rod myoids (outer side) are present. Hybridization signals were found in the cell bodies of rods and their myoid processes, suggesting that KFH-Rh mRNA is localized in rods. Figure 3(C) shows the localization of KFH-Rh mRNA in a horizontal section of fry killifish retina. It was confirmed that KFH-Rh mRNA does not exist in cone cells, so we conclude that KFH-Rh is expressed only in rods. Note that cone photoreceptor cells are arranged in a square mosaic pattern [a schematic drawing of the cone mosaic array is shown in Fig. 6(A)], as described previously by Ohki & Aoki (1985). Hybridization signals found in the cone mosaic array suggest that rod myoids protrude into the cone layer more or less randomly.

Positive signals for KFH-R and KFH-G cRNA probes were observed in the myoid regions of principle and accessory members, respectively, of the double cones [Fig. 4(A) and Fig. 4(B)]. KFH-B and KFH-V cRNA probes reacted, respectively, with long and short single cones [Fig. 4(C) and Fig. 4(D)]. The hybridization signals were localized in the myoid regions of the cone inner segments, as shown previously for other cone types (Raymond *et al.*, 1993; Stenkamp *et al.*, 1996), strongly supporting the proposal that visual pigments encoded by KFH-R, -G, -B and -V cDNAs are expressed in killifish cones, as indicated in Fig. 4(A–D). However, the subcellular localizations of KFH-R and KFH-G mRNAs are somewhat different (Fig. 5). The principle member of double cones has a longer inner segment and a shorter myoid than the accessory, and hybridization signals of KFH-R mRNA are observed only in the distal region of the myoids of the principle member. On the other hand, signals of KFH-G mRNA are localized only in the

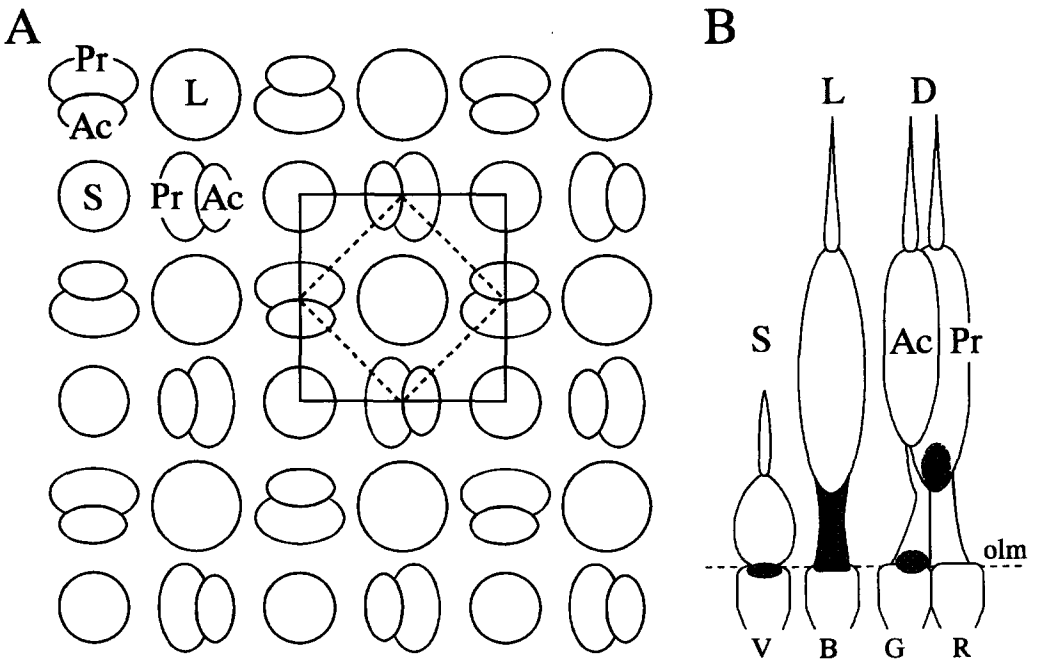

FIGURE 6. A schematic drawing of the arrangement (A) and morphologies (B) of killifish cone photoreceptor cells. Box indicates repeat unit. Subcellular localizations of opsin mRNAs are shaded. Abbreviations as in Fig. 3.

proximal myoids of the accessory member. Such differences in subcellular localization have not been reported for goldfish opsin mRNAs. A schematic drawing of killifish cone cells and the subcellular localization of hybridization signals is shown in Fig. 6(B).

Relationship between cell types and visual pigment gene expression

The relationship between cell type and the visual pigment genes expressed in killifish shows a clear correspondence to that of goldfish. That is, rod cells and principle and accessory members of double cones express the visual pigment genes belonging to the *deuterope*, *protope* and *quartope* groups, respectively, and the long and short single cones (corresponding to short single and miniature short single cones, respectively, in goldfish) express the visual pigments of the *quintope* and *tritope* groups, respectively.

The cone photoreceptor cells of many teleost fishes are well known to be morphologically distinguishable as principle and accessory members of double cones, long single cones and short single cones (Munz & McFarland, 1977). Also, a correlation has been shown between outer segment morphology and spectral sensitivity of cone photoreceptor cells in fishes (Loew & Lythgoe, 1978). Teleost fishes are classified into three major taxonomic groups: *Clupeichthyes*, *Mesichthyes* and *Percichthyes* (Goshline, 1971). The killifish (*Atheriniformes*) belongs to the *Mesichthyes* and the goldfish (*Cypriniformes*) is a member of the *Clupeichthyes*, so killifish and goldfish are not close phylogenetically, as suggested also by the fact that the deduced amino acid sequence of KFH-Rh is more closely related to the rhodopsin of the sand goby (*Percichthyes, Perciformes*) than to that of the goldfish (Fig. 2). The primary structures of killifish visual pigments and their cell-specific expression pattern show similar correspondence to those of goldfish, suggesting strongly that photoreceptor morphology, spectral sensitivity and visual pigment gene group are closely related throughout the teleosts.

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Acknowledgements—This work was partly supported by a Grant-in-Aid from the Japanese Ministry of Education, Culture and Welfare, and by SUNBOR. Takunori Satoh is supported by JSPS Research Fellowships for Young Scientists. The authors thank Prof. Pamela A. Raymond (The University of Michigan) for helpful suggestions, and Dr. Ian G. Gleadall for comments on the manuscript.